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## Molecular examinations of *Babesia microti* in rodents and rodent-attached ticks from urban and sylvatic habitats in Germany

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**Abstract:** Small mammals serve as reservoir hosts for tick-borne pathogens, especially for those which are not transmitted transovarially in ticks - such as *Babesia microti*. Molecular investigations on the prevalence of *B. microti* in wild small mammals and on attached ticks from differently structured areas may provide information on the circulation of *B. microti* in different ecological niches. In 2012 and 2013, 622 rodents (396 *Myodes glareolus*, 178 *Apodemus flavicollis*, 36 *Apodemus sylvaticus*, 4 *Apodemus agrarius*, 7 *Microtus arvalis*, 1 *Microtus agrestis*) were captured from three differently structured habitats (urban, sylvatic, recultivated) in Germany. Attached ticks were collected from 449 small mammals (3250 *Ixodes ricinus*, 7 *Ixodes trianguliceps*, 133 *Dermacentor reticulatus*). A representative selection of a maximum of 5 ticks per developmental stage and species per 30 rodents of each species, location and year resulting in 965 ticks was further investigated. DNA was extracted from tick, blood and spleen samples, and tested by PCR for the partial 18S rRNA gene of *B. microti* with subsequent sequencing. The prevalence was significantly higher in rodents from the sylvatic site (4.6%) than in rodents captured at both other sites (0-0.6%) ( $\chi^2 = 11.95$ ;  $p = 0.00125$ ). Body and spleen weight of infected *M. glareolus* from the sylvatic site were significantly higher compared to those from non-infected individuals from that site ( $p = 0.00288$  and  $p = 0.00017$ , respectively). *Babesia microti* DNA was detected in 3 out of 965 attached ticks (0.3%; 95%CI: 0-1) from all sites, but they derived exclusively from rodents captured at the sylvatic site. At the same site, *I. ricinus* nymphs (7.7%; 95%CI: 1-25.3) were significantly more often infected than *I. ricinus* larvae (0%; 95%CI: 0-1.3) ( $\chi^2 = 26.72$ ;  $p < 0.0001$ ). The majority of positive rodents was also found at that site. *I. trianguliceps* occurred exclusively and the majority of *M. glareolus* at that site. Thus, it may be assumed that the circulation of *B. microti* is more efficient where this tick species and voles exist sympatrically than in areas with a predominant occurrence of *Apodemus* species.

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**Molecular examinations of *Babesia microti* in rodents and rodent-attached ticks from urban and sylvatic habitats in Germany**

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## Abstract

Small mammals serve as reservoir hosts for tick-borne pathogens, especially for those which are not transmitted transovarially in ticks - such as *Babesia microti*. Molecular investigations on the prevalence of *B. microti* in wild small mammals and on attached ticks from differently structured areas may provide information on the circulation of *B. microti* in different ecological niches. In 2012 and 2013, 622 rodents (396 *Myodes glareolus*, 178 *Apodemus flavicollis*, 36 *Apodemus sylvaticus*, 4 *Apodemus agrarius*, 7 *Microtus arvalis*, 1 *Microtus agrestis*) were captured from three differently structured habitats (urban, sylvatic, recultivated) in Germany. Attached ticks were collected from 449 small mammals (3250 *Ixodes ricinus*, 7 *Ixodes trianguliceps*, 133 *Dermacentor reticulatus*). A representative selection of a maximum of 5 ticks per developmental stage and species per 30 rodents of each species, location and year resulting in 965 ticks was further investigated. DNA was extracted from tick, blood and spleen samples, and tested by PCR for the partial 18S rRNA gene of *B. microti* with subsequent sequencing. The prevalence was significantly higher in rodents from the sylvatic site (4.6%) than in rodents captured at both other sites (0-0.6%) ( $\chi^2=11.95$ ;  $p=0.00125$ ). Body and spleen weight of infected *M. glareolus* from the sylvatic site were significantly higher compared to those from non-infected individuals from that site ( $p=0.00288$  and  $p=0.00017$ , respectively). *Babesia microti* DNA was detected in 3 out of 965 attached ticks (0.3%; 95%CI: 0-1) from all sites, but they derived exclusively from rodents captured at the sylvatic site. At the same site, *I. ricinus* nymphs (7.7%; 95%CI: 1-25.3) were significantly more often infected than *I. ricinus* larvae (0%; 95%CI: 0-1.3) ( $\chi^2=26.72$ ;  $p<0.0001$ ). The majority of positive rodents was also found at that site. *I. trianguliceps* occurred exclusively and the majority of *M. glareolus* at that site. Thus, it may be assumed that the circulation of *B. microti* is more efficient where this tick species and voles exist sympatrically than in areas with a predominant occurrence of *Apodemus* species.

## Keywords

Tick-borne pathogen; *Babesia microti*; *Ixodes ricinus*; *Ixodes trianguliceps*; Rodent.

## Introduction

Small mammals serve as hosts for ixodid ticks. Microtine voles have been discussed as reservoir hosts for the intraerythrocytic protozoan *Babesia microti* (phylum Apicomplexa, order Piroplasmida) [Gray et al. 2002]. It was first described in a vole near Lisbon in 1910 as *Smithia microtia* (*Microtus incertus*, now *Microtus arvalis* subspecies *incertus*, the common vole [Walter 1981]. *Babesia microti* has since been detected in small mammals, humans and hard ticks from Europe and the USA [Kogut et al. 2005, Hildebrandt et al. 2007, Silaghi et al. 2012, Hersh et al. 2012]. Human babesiosis caused by *B. microti* displays subclinical to fatal malaria-like symptoms. It primarily occurs in the USA [Vannier and Krause 2012]. In Europe however, severe cases of human babesiosis have mainly been caused by *Babesia divergens*-like parasites in immunocompromised patients, but cases have also been reported in immunocompetent patients [Hildebrandt et al. 2013, Mathis et al. 2006, Martinot et al. 2011]. Transplacental transmission and transmission via tick bites have been described before [Vannier and Krause 2009]. However the first and so far only autochthonous human case caused by *B. microti* in Europe was transmitted by blood transfusion [Hildebrandt et al. 2007]. While transovarial transmission in ticks was confirmed for *B. divergens* and other *Babesia* species, experimentations have failed to demonstrate transovarial transmission for *B. microti* [Gray et al. 2010]. Therefore mammalian hosts are essential for its maintenance in natural transmission cycles [Gray et al. 2002]. As information on the enzootic life cycle of *B. microti* is still incomplete, the objectives of this study were to detect and compare *B. microti* prevalences in wild rodents and attached ticks as well as to assess their role in the circulation of this protozoan at ecologically different habitats in Germany.

## Material and Methods

### *Collection of wildlife samples*

In 2012 and 2013 small mammals were captured at three differently structured sites in Germany. The urban site “R” (7.4 ha, 49°00′55.72″N, 12°05′08.89″E) is a small park near the city centre of Regensburg. It is surrounded by walls and high traffic roads. The park is highly frequented by visitors.

Large wild animals are absent. Sylvatic site “T” (641 ha, 48°06′36.42″N, 10°34′33.40″E), located near Tussenhausen in Bavaria, is a large forest area (641 ha). This mixed forest is dominated by beeches, oaks and spruces. Different wild animal species are present and the anthropogenic influence is low. Forestry is not cultivated extensively. Therefore, there is little to no interaction between wild and domestic animals and humans [Forstdirektion Oberbayern]. The recultivated site “S” is subdivided in three locations (51°15′32.2″N, 12°21′02.5″E, 51°17′01.3″N, 12°21′00.6″E, 51°26′97.2″N, 12°32′25.6″E), formerly described as site E-G, which surround an artificially created lake “Lake Cospuden”, near Leipzig, Saxony [Silaghi et al., 2012]. The lake belongs to a recultivated area, which was created out of a former brown coal mining area. Today, the surroundings of this lake are of public health interest as it is the largest recreational area near Leipzig (<http://www.leipzigerneuseenland.de/>). Bushes and trees less than 20 years old characterize this site. Nevertheless a high diversity of large and small wild mammals is present. Details of the investigated sites have been described previously [Silaghi et al. 2012, Obiegala et al. 2014, Overzier et al. 2013]. Small mammals were trapped using Sherman® live animal traps (H. B. Sherman Traps, Inc., Tallahassee, Fla. U.S.A.) (official permit Site R1: 55.1-8646.4-140, Site T: 55.1-8646-2/30, Site S: AZ 36.11-36.45.12/4/12-001). Traps, baited with apple slices, were placed for at least two consecutive nights per month and site and were checked twice a day. Collected animals were anaesthetized with CO<sub>2</sub> and then euthanized by cervical dislocation. Blood was taken by heart puncture. Animals were sexed, weighed and necropsy was performed to obtain spleen samples. All small mammals were identified by the use of taxonomic keys [Stresemann 1989]. Further, a conventional PCR targeting the *cytochrome b* gene [Parson et al. 2000] yielding an amplicon of 354 base pairs was performed to verify morphological identification for a randomly selected number of 69 individuals as described [Obiegala et al. 2014].

### ***DNA Extraction and PCR analysis***

DNA was extracted from ticks, blood and spleen samples from small mammals with the Maxwell® 16 LEV blood DNA kit (Promega GmbH, Mannheim, Germany) and the corresponding Maxwell® 16

System (Promega GmbH) as described [Obiegala et al. 2014]. PCR amplification of an *18S rRNA* *Babesia* gene fragment (411-452 bp) was performed using a previously published PCR protocol [Casati et al. 2006] with modifications as described [Schorn et al. 2011]. Amplicons were detected by agarose gel electrophoresis and purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) for sequencing at Eurofins MWG Operon, Ebersberg, Germany. Sequencing was performed with forward and reverse primers used for PCR amplification. Sequences were analysed with the Chromas Lite program (Technelysium Pty Ltd, South Brisbane, Australia) and aligned to available sequences in the GenBank with BLASTn (National Center for Biotechnology Information, Bethesda MD, USA) [Schorn et al. 2011].

### **Statistical analysis**

Graph Pad Software (Graph Pad Software, Inc., San Diego, Ca., USA) was used for the calculation of confidence intervals (CI 95%) of prevalence rates with the Clopper and Pearson method. Pearson's chi-squared test with a type I error  $\alpha$  of 0.05 and Fisher's exact test for the comparison of sample sizes  $< 5$  were used to compare *B. microti* prevalences. Comparisons of multiple values were adjusted with the Bonferroni correction. To compare body and spleen weight of infected and non-infected *M. glareolus* at the sylvatic site the one-tailed Wilcoxon-Mann-Whitney-Test with  $\alpha=2.5$  was used. Results with p values  $< 0.05$  were considered to be positive.

## **Results**

### **Collection of wildlife samples**

Altogether 622 rodents of six species (4 *Apodemus agrarius*, 36 *A. sylvaticus*, 178 *A. flavicollis*, 7 *Microtus arvalis*, 1 *Mi. agrestis*, 396 *M. glareolus*) were collected. From these animals 443 blood and 585 spleen samples were available for further examination (Table 1). Altogether 3,391 ticks of three species (7 *Ixodes trianguliceps*, 3,250 *I. ricinus*, 133 *Dermacentor reticulatus*) were collected from 449 of the 622 small mammals. Altogether 92.1% of all collected ticks were larvae. Nymphs were exclusively collected from *M. glareolus* and *A. flavicollis*. Tick infestation rates ranged from 1 to 112

ticks with a mean infestation of 8 ticks and a median infestation of 16 ticks per small mammal. For *M. glareolus* the mean infestation rate was 4 ticks and the median infestation was 6 ticks. The mean infestation on *A. flavicollis* was 10 ticks per mouse and the median infestation was 23 per mouse. For *A. sylvaticus* the mean infestation rate was 4 ticks and the median infestation was 9. *I. trianguliceps* ticks were found on *M. glareolus* and *A. flavicollis* exclusively in the sylvatic site T. A total of 98.5% of all *D. reticulatus* ticks were collected from *M. glareolus* and only 1.5% from *A. flavicollis*. All *D. reticulatus* were found at the recultivated site S. According to the availability of different rodent and tick species, 5 ticks per tick stage and species of 30 individuals of each rodent species were selected per year and site. This resulted in 965 ticks from 186 rodents for further analysis (Table 2).

### ***Babesia* spp. detection and sequence analysis**

In total 13 out of 622 (2.1%; 95%CI: 1.2-3.6) small mammals were positive for *B. microti*. Of those, 11 rodents were from site T (4.6%; 95%CI: 2.5-8.2), two from site S (0.6%; 95%CI: 0-2.2) and none from site R1 (Table 1). The prevalence for *B. microti* was significantly higher at site T than at site R1 and site S ( $\chi^2=11.95$ ;  $p=0.00125$ ). At site T, *M. glareolus* (7.2%; 95%CI: 3.8-12.9) were significantly more often infected than *A. flavicollis* (1%; 95%CI: 0-6) ( $\chi^2=5.03$ ;  $p=0.025$ ). Body and spleen weight was significantly higher in infected ( $n_1=10$ ) than in non-infected *M. glareolus* ( $n_2=229$ ) from site T ( $U=976$ ;  $p=0.00288$ ; respectively for the spleen weight:  $U=1084.5$ ;  $p<0.001$ ) (Figure 1). The ratio between body and spleen weight was 242:1 for non-infected and 48:1 for infected *M. glareolus*. The infestation rate of ticks on infected rodents ranged from 0 to 29. Altogether, 3 out of 965 (0.3%; 95%CI: 0-1) attached ticks were positive for *B. microti* (Table 2). These ticks originated from site T (0.79%; 95%CI: 0.2-2.4) (2 *I. ricinus* nymphs, 1 *I. trianguliceps* nymph) and two of them (1 *I. ricinus*, 1 *I. trianguliceps*) were collected from *B. microti*-positive rodents (Table 2). All nymphs ( $n=2$ ) collected from infected rodents were positive for *B. microti* however none of the larvae collected from infected rodents was positive ( $n=89$ ). *Dermacentor reticulatus* ticks, regardless whether larvae or nymphs, (0%; 95%CI: 0-10.4) were negative for *B. microti*. Altogether, 16 *B. microti* sequences were obtained from 13 rodents, as 3 of those were positive in both blood and spleen. Three sequences were obtained from rodent-

attached ticks. All sequences were 100% identical to short sequences of the pathogenic *B. microti* Jena/Germany genotype (GenBank accession no. JQ880658.1, JX6273569.1). This does not mean that the sequenced parasites from this study are necessarily pathogenic. Obtained sequences were deposited in GenBank under the following accession numbers: KP055632-KP055650.

## Discussion

The prevalence of *B. microti* in small mammals from Slovenia, Great Britain, Poland and Germany ranged between 1.6% and 42% [Duh et al. 2003, Bown et al. 2008, Karbowski 2004, Pawelczyk et al. 2004, Silaghi et al. 2012]. Our findings are in line with the lower range of these previous observations (2.1%). In *Mi. agrestis*, a former study showed a prevalence of *B. microti* infection (9 %) comparable to our findings for this rodent species (14.3%) [Pawelczyk et al. 2004]. *Mi. agrestis* and *M. glareolus* (family Cricetidae) have been discussed as main reservoirs for *B. microti* [Bown et al. 2008].

Splenomegaly and a related higher body weight were observed for *B. microti*-infected *M. glareolus* compared to non-infected ones at site T. This result may be explained by the *B. microti* infection which causes splenomegaly in small mammals [Welc-Faleciak et al. 2007]. Whereas in 2011 respectively in 2003 Beck et al. and Duh et al. found *A. flavicollis* positive for *B. microti* in a high percentage (11.8-16.2%), the prevalence for this rodent species was very low in our study (0.01%). Furthermore, other *Apodemus* species such as *Apodemus sylvaticus* and *A. agrarius* were negative for *B. microti* in those previous studies [Beck et al. 2011, Duh et al. 2003]. This is in line with our findings in these two species and supports the hypothesis that mice of the species *A. agrarius* and *A. sylvaticus* are subordinately involved in the natural life cycle of *B. microti*. Additionally, *I. trianguliceps* ticks, adapted to voles [Bown et al. 2008] and present at sylvatic areas [Bown et al. 2003], have been pointed out as the main vector for *B. microti* among rodents and shrews [Nefedova et al. 2013, Randolph 1995]. Significantly more positive rodents were found at the sylvatic site T than at the recultivated (S) and urban site (R1). In this context, one out of seven *I. trianguliceps* ticks originating exclusively from the sylvatic site, where also the majority of positive voles was found, was positive for *B. microti*. The prevalence rates in *I. ricinus* ticks from the present study are comparable



to the lower prevalence (0.003-10.1%) from other European countries such as Slovenia, the Netherlands, France, Belgium and Switzerland [Foppa et al. 2002, Lempereur et al. 2011, Bonnet et al. 2014, Duh et al. 2001, Wielinga et al. 2009]. All three positive ticks (nymphs) were attached to rodents captured at the sylvatic site T. No transovarial transmission for *B. microti* in ticks was described before which may explain that none of the 846 analysed larvae, regardless of the tick species, was positive [Homer et al. 2000]. At site T, two out of three *B. microti*-positive nymphs (1 *I. ricinus*, 1 *I. trianguliceps*) derived from *B. microti*-positive rodents. This result underlines particularly the potential reservoir role of *M. glareolus* for *B. microti* in sylvatic habitats. However none of 89 larvae deriving from positive rodents was positive for *B. microti*. The infection rates of fully engorged larvae deriving from infected rodents, is higher than of larvae which are less engorged [Homer et al. 2000]. None of the larvae in this study detached naturally which could be an explanation for this result. Moreover, 5 rodents were solely positive in spleen and not in blood. According to our results and to those from former studies [Wójcik-Fatla et al. 2012], *I. ricinus* may serve as a bridge vector [Silaghi et al. 2012; Overzier et al. 2013]. On the contrary, we cannot confirm that *D. reticulatus* ticks may serve as bridge vectors for *B. microti* even though its role has also been discussed before [Wójcik-Fatla et al. 2012]. The sequences obtained from both ticks and rodents all showed a 100% identity to each other and to sequences deposited under following GenBank accession no.: JQ880658.1, JX6273569.1. This sequence belongs to the pathogenic *B. microti* Jena/Germany genotype which was published in the context of the first autochthonous human babesiosis case in Europe [Hildebrandt et al. 2007]. The existence of reservoir hosts and bridge vector ticks infected with this particular strain may constitute a risk to human health at the investigated sylvatic site, even though only one human case has been reported in Europe thus far.

The life cycle of *B. microti* seems adapted to an environment with sympatric existence of voles and *I. trianguliceps* ticks. Besides, according to our results, *Apodemus* species seem of secondary importance in the transmission of *B. microti*.

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**Table 1 Prevalences of *Babesia microti* in spleen and/or blood samples from small mammals per site and year, detected by PCR**

Number of mammals positive for <i>Babesia microti</i> (%)							
Mammal	Site S		Site T		Site R1		Total
Species	2012	2013	2012	2013	2012	2013	

<i>Myodes glareolus</i>	1/229 (0.004)	0/28 (0)	7/131 (5.3)	3/8 (37.5)	-	-	<b>11/396 (0.03)</b>
<i>Apodemus sylvaticus</i>	-	-	-	-	0/22 (0)	0/14 (0)	<b>0/36 (0)</b>
<i>Apodemus agrarius</i>	0/4 (0)	-	-	-	-	-	<b>0/4 (0)</b>
<i>Apodemus flavicollis</i>	0/57 (0)	0/22 (0)	1/84 (1.2)	0/15 (0)	-	-	<b>1/ 178 (0.01)</b>
<i>Microtus arvalis</i>	1/7 (14.3)	-	-	-	-	-	<b>1/7 (14.3)</b>
<i>Microtus agrestis</i>	0/1 (0)	-	-	-	-	-	<b>0/1 (0)</b>
<hr/>							
<b>Total</b>	<b>2/298 (0.01)</b>	<b>0/50 (0)</b>	<b>8/215 (3.7)</b>	<b>3/23 (13.4)</b>	<b>0/22 (0)</b>	<b>0/14 (0)</b>	<b>13/622 (0.02)</b>
	<hr/>						
	<b>2/348 (0.6)</b>		<b>11/238 (4.6)*</b>		<b>0/36 (0%)</b>		

\*statistically significantly higher value ( $\chi^2=12.1398$ ;  $p= 0.0005$ ) compared to the other sites in combined years 2012 and 2013, tested by the Chi-square test ( $\alpha=0.05$ ) and the Bonferroni correction for multiple values

1

2 **Table 2 Developmental stage and sex of attached ticks per species and per location collected from small mammals captured in 2012 and 2013 from three**  
 3 **sites S<sup>1</sup>, T<sup>2</sup>, R1<sup>3</sup>, and number of *B. microti* positive ticks in a selected number of investigated ticks.**

Number of collected ticks (number of <i>B. microti</i> positive ticks/number of investigated ticks )								
Tick species	Site S <sup>1</sup>		Site T <sup>2</sup>		Site R1 <sup>3</sup>		Total ticks per species	
	Nymphs	Larvae	Adult <sup>c</sup>	Nymphs	Larvae	Nymphs	Larvae	
<i>I. ricinus</i>	180 (0/73)	1740 (0/377)	-	44 (2*/22)	1132 (0/352)	3 (0/3)	151 (0/91)	<b>3250 (2/918)</b>
<i>I. trianguliceps</i>	-	-	1 (0/1)	5 (1**/5)	2 (0/1)	-	-	<b>8 (1/7)</b>
<i>D. reticulatus</i>	35 (0/15)	98 (0/25)	-	-	-	-	-	<b>133 (0/40)</b>
<b>Total</b>	<b>2053 (0/490<sup>d</sup>)</b>			<b>1184 (3/381<sup>e</sup>)</b>		<b>154 (0/94<sup>f</sup>)</b>		<b>3391 (3/965)</b>

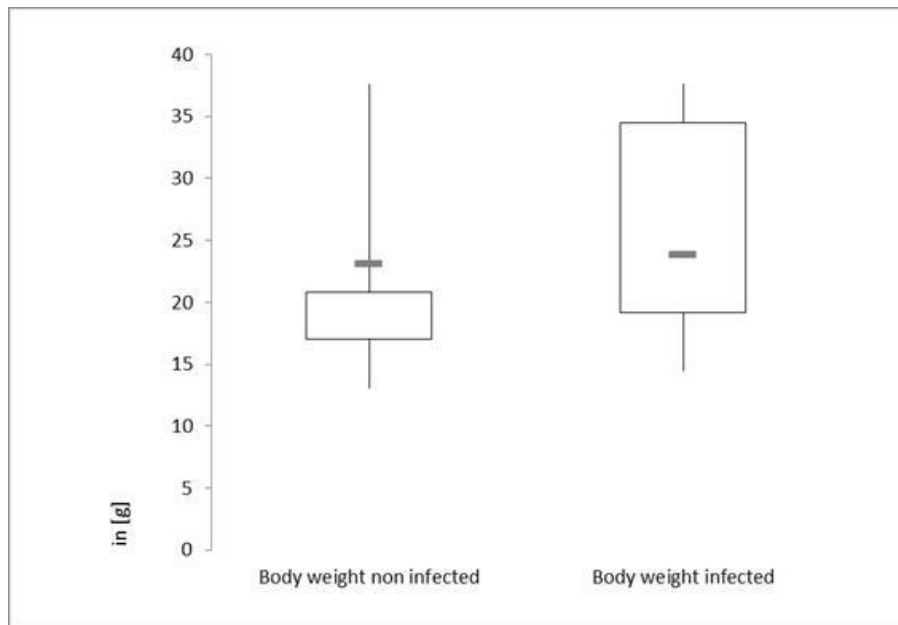
12 <sup>1</sup>recultivated site; <sup>2</sup>sylvatic site; <sup>3</sup> urban site, <sup>c</sup>female tick from site T; <sup>d</sup>number of ticks selected from 77 rodents; <sup>e</sup>number of ticks selected from 79 rodents;

13 <sup>f</sup>number of ticks selected from 30 rodents, \*one of those was collected from an infected *Myodes glareolus*, \*\*collected from an infected *M. glareolus*;



**Figure 1** Box plots of body (a) and spleen weights (b) from *Myodes glareolus* from site T, which were PCR negative (n=129; number of females= 71; number of males=58) or positive (n=10; number of females=4; number of males=6) for *Babesia microti*.

(a)



(b)

